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(54) **Pharmaceutical compositions comprising at least one tetrahydropyrimidine derivative (THP(A) or THP(B))**

Mindestens ein Tetrahydropyrimidinderivat (THP(A) oder THP(B)) enthaltende pharmazeutische Zusammensetzungen

Compositions pharmaceutiques comprenant au moins un dérivé de tétrahydropyrimidine (THP(A) ou THP(B))

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- **PATENT ABSTRACTS OF JAPAN, unexamined applications, C field, vol. 14, no. 310, July 04, 1990 THE PATENT OFFICE JAPANESE GOVERNMENT page 40 C 736; & JP-A-2 104 677 (KOEI CHEM)**
- **CHEMICAL ABSTRACTS, vol. 109, no. 23, December 05, 1988 Columbus, Ohio, USA L. INBAR et al. "The structure and biosynthesis of new tetrahydropyrimidine derivatives in actinomycin D producer Streptomyces parvulus. Use of carbon-13- and nitrogen-15-labeled L-glutamate and carbon-13 and nitrogen-15 NMR spectroscopy", page 379, right column, abstract-no. 208 058b**

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Description

The present invention relates to pharmaceutical compositions comprising purified tetrahydropyrimidine derivatives. More particularly, the invention relates to tetrahydropyrimidine derivatives useful for DNA protection from damaging by DNA-binding drugs, chemical carcinogens and mutagens and from irradiation.

Actinomycins, a class of antitumor antibiotics used in cancer chemotherapy, prevent cell replication by positioning themselves between planar base pairs of DNA, thus preventing the unwinding of the double helix, and also inhibit transcription, i.e., DNA-dependent RNA synthesis, both in vivo and in vitro. The transcriptional inhibition in vivo or in vitro is thought to occur through binding of the drug to DNA and blockage of the RNA polymerase during elongation. Intercalators of DNA, such as actinomycin D and daunomycin, were shown to affect the DNA secondary structure.

Actinomycin D (hereinafter Act D), also called dactinomycin, and adriamycin are well known antitumor antibiotics. However, their high toxicity prevents their general use in cancer chemotherapy. It would be highly desirable to lower the toxicity of Act D and of other antitumor and cytotoxic antibiotics, thus allowing their effective use in antitumor therapy.

Two previously unknown metabolites tetrahydropyrimidine derivatives, 2-methyl-4-carboxy-5-hydroxy-3,4,5,6-tetrahydropyrimidine (designated THP(A) hereinafter) and 2-methyl-4-carboxy-3,4,5,6-tetrahydropyrimidine (designated THP(B) hereinafter), were found by the inventors of the present patent application to accumulate intracellularly in Act D-producing gram-positive actinomycete, *Streptomyces parvulus*, and were identified by in vivo and in vitro NMR studies, but were neither isolated nor purified (Inbar and Lapidot, 1988a, 1988b, 1991).

THP(B) in purified form had earlier been extracted from the culture of *Ectothiorhodospira halochloris*, an extremely halophilic bacteria. It was called "ectoine" and suggested to serve an osmoregulatory function (Galinski et al., 1985). Crystals of THP(B).CH₃OH were crystallized from water-free methanol (Schuh et al., 1985). Ectoine, a cyclic amino acid, was later found in a variety of different halophilic and halotolerant eubacteria, which are organisms of extremely saline habitats, such as *Halomonas elongata*, *Deleya halophila*, *Halomonas halmophila*, *Bacterium Ba*, *Vibrio costicola* and *Brevibacterium linens*. The natural environment of these microorganisms - salt or soda lakes, solar saltworks, coastal lagoons or sea water, is characterized by a high ionic concentration and low water activity. For the maintenance of osmotic equilibrium, most halophilic eubacteria as well as yeasts, algae and fungi, accumulate inorganic ions, such as K⁺, Na⁺ and Cl⁻ and neutral non-ionic organic molecules of low molecular weight, represented by the following major classes: polyols, sugars, some amino acids, betaines and ectoines. These solutes are called "compatible solutes" because they compensate the osmotic pressure and, by definition, are compatible with the metabolism of the cell. Apart from their purely osmotic function, compatible solutes, including ectoine, also exhibit protective effect on enzymes, e.g. against heating, freezing and drying (Wohlfarth et al., 1990; Regev et al., 1990; Peters et al., 1990; Galinski and Lippert, 1991). Japanese Patent Application published under No. 3031265, of Takeda Chemical Ind., discloses the chemical synthesis of ectoine and adds that it is useful as intermediate for medical agent, agricultural agent or organic electronic materials. None of the prior art discloses any pharmaceutical effect of THP(A) and THP(B), and particularly not their effect in protecting DNA from damage.

The inventors propose that there is a similarity in self-defence strategies in Act D-producing bacteria and halophilic eubacteria. The question of how antibiotic-producing organisms avoid suicide has been the subject of many studies. The target for drug action is known to be refractory to the endogenous antibiotics in the various organisms that produce them, although in many cases, the basis of such resistance has not been fully established. Only a few mechanisms of self-modification of antibiotic target sites have been characterized. In cases in which the antibiotic is involved in protein synthesis, self-protection depends on specific methylation of the ribosomal RNA target. However, interference with the antibiotic's ability to bind at the DNA target has not yet been achieved.

Act D is a potent inhibitor of DNA-dependent RNA synthesis. Since *S. parvulus* produces Act D during the normal course of its development, synthesizing RNA while simultaneously producing the antibiotic, it seems possible that resistance of the transcriptional machinery to Act D might be a characteristic of the cells.

Similarly, the resistance of the transcriptional machinery in halophilic microorganisms to high electrolyte concentrations might be an intrinsic feature of these cells. Since DNA at physiological pH is a very highly charged polyanion and so is surrounded by cations, the binding of proteins to DNA results in their displacement from the surface area of the DNA. How DNA-protein interactions are buffered in vivo in halophilic bacteria against the adverse effects of fluctuations in cellular ion concentration is a challenging question in the field of osmoregulation. The halophilic organisms must adopt survival strategies in order to avoid structural disturbances of cellular macromolecules at high intracellular potassium ion concentrations. Various antibiotic-producing strains of the *Streptomyces* genus were isolated from soil after being exposed to localized changes in soil salinity. It is herein proposed that similar mechanisms exist in these microorganisms to regulate responses both to antibiotic and osmotic stress.

Intracellular effectors were observed using NMR techniques to elucidate the mechanisms of self-defence in antibiotic-producing organisms. In the study of the control mechanism of Act D biosynthesis by *S. parvulus*, THP(A) and THP(B) were identified and found to accumulate intracellularly (Inbar and Lapidot, 1988a). Labeling experiments established that the THPs are not precursors of Act D (Inbar and Lapidot, 1988b). By NMR and X-ray crystallography of

isolated and purified THP(A) and THP(B) according to the invention, it is shown here that the THPs form zwitterionic molecules with the half-chair conformation (Fig. 1).

It was found that THP(A) and THP(B) are present not only in *S. parvulus*, but also in closely related Act D-producing microorganisms, such as *Streptomyces chrysomallus* and *Streptomyces antibioticus*, as shown by ¹³C NMR experiments that revealed clearly that THP(A) and THP(B) were produced in these strains during Act D synthesis. The finding of THPs in several *Streptomyces* producing Act D, their relatively high intracellular concentrations during Act D synthesis, and the identity of the time of onset of their synthesis with that of Act D synthesis, indicate that they may function in the self-defense mechanism of Act D-producing organisms. Furthermore, using an *in vitro* transcription system (Kadesch and Chamberlin, 1982) in which RNA polymerase II was completely inhibited by Act D, THP(A) and THP(B) were found to act in concert to partially restore RNA polymerase activity.

It was thus found, in accordance with the present invention, that THP(A) alone or THP(A) and THP(B) in combination can protect DNA from digestion by restriction endonucleases. They also enable efficient growth of *E. coli* under high salt concentrations or in the presence of adriamycin, conditions which otherwise prevent *E. coli* growth. Furthermore, THP(A) and THP(B) function synergistically to prevent DNA - Act D complex formation. THP(A) alone or a mixture of THP(A) and THP(B) further have a direct effect on RNA transcription in the presence of Act D, as demonstrated in an *in vitro* transcription assay.

It is an object of the present invention to provide pharmaceutical compositions comprising THP(A) and/or THP(B), and a pharmaceutically acceptable carrier. These compositions will particularly protect DNA in non-tumor tissues from damaging by DNA-binding drugs, such as toxic antitumor drugs, or by chemical carcinogens and mutagens or irradiation, such as UV irradiation.

The pharmaceutical compositions of the invention comprise as active ingredient a tetrahydropyrimidine derivative selected from 2-methyl-4-carboxy-5-hydroxy-3,4,5,6-tetrahydropyrimidine (THP(A)), a salt, a 5-ether or a 5-ester thereof, and/or 2-methyl-4-carboxy-3,4,5,6-tetrahydropyrimidine (THP(B)) or a salt thereof, and a pharmaceutically acceptable carrier.

Other objects and advantages of the invention will become apparent from the disclosure of the invention.

THP(A) and THP(B) can be obtained synthetically or can be isolated and separated in purified form from natural sources, such as cell extracts of Act D-producing microorganisms of the *Streptomyces* species, e.g. *S. parvulus*, *S. chrysomallus*, or *S. antibioticus* and mutants thereof. THP(B) alone can be isolated and purified from halophilic and halotolerant bacteria under salt stress, such as bacteria of the genus *Ectothiorhodospira*, e.g. *E. halochloris*, *E. halophila* and mutants thereof, or from heterotrophic halophilic eubacteria of the family *Halomonadaceae*. THP(A) alone can be produced by soil microorganisms of the *Streptomyces* species, e.g., *S. clavuliferus*, *S. griseus* and mutants thereof, under low salt stress.

THP(A) and THP(B) can be isolated and purified from cell extracts of an Act D-producing *Streptomyces* strain by a method comprising the following steps:

- (a) cultivating said *Streptomyces* strain in a suitable growth medium;
- (b) harvesting the cells and preparing cell extracts from the washed cells;
- (c) separating the tetrahydropyrimidine derivatives THP(A) and THP(B) from accompanying carbohydrates, polyols, amino acids, peptides and proteins by several column chromatography separation steps;
- (d) subjecting the solution containing the two tetrahydropyrimidine derivatives to ion exchange column chromatography; and
- (e) eluting each tetrahydropyrimidine derivative with a different eluent, whereby substantially pure THP(A) and THP(B) are obtained.

The Act D-producing *Streptomyces* strain preferably used in the invention is *S. parvulus*, more particularly *S. parvulus* ATCC 12434, freely available from ATCC.

Cell extracts are prepared by methods known in the art, e.g. by suspending washed cell pellets in water and heating at 100°C or by perchloric acid procedure. The cell extracts are then first mixed with acetic acid before subjecting them to separation procedures. First, carbohydrates and polyols are removed, e.g., by water; then amino acids are removed by anion exchange chromatography, and residues of intracellular peptides and proteins are removed by Sephadex®. The separation of THP(B) from THP(A) in step (d) is then carried out on a Dowex® 50W (NH₄⁺ form) column and in step (e), THP(A) is eluted with water and THP(B) with 3 M ammonium hydroxide.

As an alternative THP(A) can be isolated and purified from cell extracts of a *Streptomyces* strain isolated from soil by a method similar to that described above and which comprises the following steps:

- (a) cultivating said *Streptomyces* strain in a suitable growth medium in 0.25M-0.5M NaCl;
- (b) harvesting the cells and preparing cell extracts from the washed cells;
- (c) separating THP(A) from accompanying carbohydrates, amino acids and proteins by several column chroma-

topography separation steps;

(d) subjecting the solution containing THP(A) to ion exchange column chromatography; and

(e) eluting the THP(A) with water, whereby substantially pure THP(A) is obtained.

The soil *Streptomyces* strains preferably used in the invention are *S. clavuligerus*, more particularly *S. clavuligerus* NRRL 3585, freely available from NRRL, and *S. griseus*, particularly *S. griseus* ATCC 10137, freely available from ATCC.

The salts of THP(A) and THP(B) to be used in the pharmaceutical compositions of the invention are physiologically acceptable salts, formed both with inorganic and organic bases. The 5-ethers of THP(A) include lower alkyl ethers and ethers formed with sugar moieties, e.g., nucleoside analogues, and are prepared by reaction with the suitable alcohols by methods known in the art. The 5-esters of THP(A) include lower alkanoyl esters prepared by reaction with suitable lower alkanic acids.

THP(A) or THP(B) or the combination thereof may be used for protecting DNA in non-tumor tissues from damage by a DNA-binding DNA-binding drug in patients undergoing treatment with the drug. The DNA-binding drug may be any of the antitumor antibiotics presently used in cancer chemotherapy, e.g. dactinomycin (Act D), daunorubicin, doxorubicin (adriamycin), bleomycin, mitomycin and the like. Since these antitumor antibiotics are toxic to normal tissue, THP(A) or THP(B) or the combination of THP(A) and THP(B) will decrease their toxic effects. Other antitumor antibiotics not currently used in cancer chemotherapy due to their high toxicity, such as the anthracycline antibiotics cinerubins A and B, might have their toxicity reduced by THP(A), THP(B) or by THP(A) + THP(B), thus allowing their future use in antitumor therapy.

THP(A), THP(B) or the combination thereof may also be useful for protection of DNA from chemical carcinogens and mutagens, including irradiation.

THP(A) and THP(B) are not toxic to mammals. When administered to mice in doses of 1, 10 and 100 mg/100 g body weight, no toxic effects were found. THP(A) and THP(B) also do not have antibiotic activity, as shown in tests carried out on several cultures of gram positive and gram negative bacteria.

THP(A) or a salt, a 5-ether or a 5-ester thereof, and THP(B) or a salt thereof are formulated into pharmaceutical compositions by mixing therapeutically effective amounts of said compounds with a pharmaceutically active carrier. Additives, such as stabilizers, etc., can be added to the composition.

Pharmaceutical compositions for any suitable mode of administration are included in the invention, but preferred are single dose intravenous injections and intravenous infusions.

The molar ratio between THP(A) and THP(B) in the pharmaceutical compositions of the invention comprising both may vary. Preferably, the pharmaceutical compositions of the invention will comprise THP(A) and THP(B) in equimolar amounts.

The effective amounts of THP(A) and THP(B) alone or in combination in the compositions of the invention will vary according to the patient's condition, the severity of the disease and the amount of antitumor drug administered to him. In one embodiment, they will be administered in excess amount to the antitumor drug.

The pharmaceutical compositions of the invention may be administered prior to, together with, or preferably subsequently to, the administration of the antitumor antibiotic. In effective amounts they will decrease the toxic effects of the antitumor drug.

The invention is illustrated by way of example only, with reference to the accompanying drawings in which:

Figure 1 depicts the (500-MHz)¹H NMR spectra of (a) purified THP(B) and (b) purified THP(A).

Figure 2 depicts the molecular structures and stereochemical configurations (S form) of THP(A) and THP(B).

Figure 3 shows the proton NMR spectra (7.6-8.4ppm; 500 MHz) of: (a) an Act D-d(ATGCAT) mixture at a ratio of 0.6:1; (b) after addition of THP(B) to the mixture at a ratio of 0.6:1:30; and (c) after addition of THP(A) to the mixture in (b) at a ratio of 0.6:1:30:30. Spectra were obtained in D₂O phosphate buffer solution, pH 7 at 10°C using solvent suppression by irradiation during acquisition delay. These experiments were done using a 4.0mM stock solution of Act D and a 100mM stock solutions of THP(A) and THP(B) in phosphate buffer. For each ¹H NMR experiment 1.8 mg d(ATGCAT) in 0.45ml phosphate buffer was used.

Figure 4 shows the base aromatic proton region spectra (at 500MHz) of Act D-d(ATGCAT) mixtures at various drug:DNA ratios as indicated in the plot (a-e), and from d(ATGCAT), THP(A) and THP(B) mixtures at molar ratios of 1:30:30 and Act D at various drug:DNA ratios, as indicated in the plot (a'-e'). Expanded views of (a) and (a') are shown in spectra (A) and (A'), respectively. The spectra were obtained at pH 7 (as in Fig. 3). The proton resonances of the Act D-d(ATGCAT) are indicated in the spectra: adenosine (internal) H2 proton at 8.03 and 8.00 ppm, adenosine (terminal) H2 protons at 7.86ppm, and guanosine H8 protons at 7.70 and 7.63ppm. The chemical shifts assignments of Act D-d(ATGCAT) were as in Brown et al. (1984).

Figure 5 is a radiogram showing the inhibition of RNA polymerase II transcription by Act D and its resumption due to the addition of THP(A) and THP(B). The constituents of each lane are indicated in Table 6. Relative intensities of the bands were determined as in Table 6. The concentration of the DNA was 71.4 μM.

Figure 6 illustrates reversal of adriamycin inhibition of DNA synthesis due to THPs. It depicts a schematic presentation of percent $[^3\text{H}]$ thymidine incorporated versus adriamycin concentration according to the table below the scheme. Empty squares - $[^3\text{H}]$ thymidine incorporation versus adriamycin concentration. Filled squares - $[^3\text{H}]$ thymidine incorporation versus adriamycin concentration in the presence of THPs (100 $\mu\text{g}/\text{ml}$).

Figure 7 illustrates reversal of actinomycin inhibition of transcription due to THPs using the HeLa WCE system. Transcription under pulse chase conditions was performed as described in Example 6. The DNA was preincubated with THPs (A+B) at increasing concentrations for 20 min at 30°C , actinomycin D was then added at a concentration of 0.4 $\mu\text{g}/\text{ml}$. Following 20 min of incubation, WCE (10 μl) was added for incubation of 20 min at 30°C . Radioactive nucleotide mix was then added for a pulse of 5 min followed by addition of chase mix containing 10 mM CTP. Transcription proceeded for 30 min at 30°C and was then stopped. RNA was extracted and analyzed as described in Example 6. Lane M - molecular weight markers. Lane C - control reaction without adriamycin or THPs. Lanes of reaction with 0.4 $\mu\text{g}/\text{ml}$ actinomycin D are marked (+), lanes of increasing concentrations of THPs (0, 5, 10, 20, 50, 100, 200, 500 $\mu\text{g}/\text{ml}$) are also marked above the lanes. The relative intensities of the bands were estimated as described in Example 6 and the percent is of the control (lane C).

Figure 8 shows proton-decoupled natural abundance ^{13}C NMR (126.7 MHz) spectra of extracts from *S. clavuligerus* cells grown in chemically defined medium at different salt concentrations. (A) 0.5M NaCl, (B) 0.25 M NaCl, (C) no salt. The ^{13}C peaks include the following: A. THP(A); Tre, trehalose; Glu, glutamic acid.

Figure 9 shows intracellular metabolites in *S. clavuligerus* cells grown in the chemically defined medium at various salt concentrations as calculated from the NMR spectrum shown in Fig. 8. Glutamic acid was measured by amino acid analyzer, and values for THP(A) and trehalose were computed by integration from NMR spectra.

Figure 10 shows proton-decoupled ^{13}C NMR (126.7 MHz) spectrum of extracts from *S. griseus* cells grown in chemically defined medium with 0.5M of NaCl for 66 hours. The ^{13}C peaks include the following: A - THP(A); Tre - trehalose; Glu-glutamic acid; Eth - ethanol (added for quantitative measurement of THP(A)).

Figure 11 shows proton-decoupled ^{13}C NMR (126.7 MHz) spectrum of purified THP(A) obtained from *S. griseus*. The invention is further illustrated by the following non-limiting examples:

EXAMPLES

Example 1: Isolation and purification of THP(A) and THP(B) from cell extracts of *S. parvulus* ATCC 12434.

(a) Cultivation of growth culture

S. parvulus (ATCC 12434), kept on soil culture at 4°C , was grown on NZ amine medium (Sheffield Chemicals, N.Y., USA) for 2 days at 30°C in a gyratory shaking incubator. After centrifugation and washing twice with 0.3% KCl, a suspension of the mycelium (3%) served as inoculum for the chemically defined growth medium (40 g D-fructose, 1.0 g K_2HPO_4 , 25 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 25 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 25 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 25 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.1 g L-glutamic acid/1000 ml of deionized water at pH 7.1).

(b) Cell Extract Preparation

Extracts of *S. parvulus* cells were prepared from 1 liter of *S. parvulus* growth culture. Cells were harvested by centrifugation (at 5,000 x g for 5 min) at about 4°C . Cells were washed twice with 0.2% KCl solution to remove traces of culture medium. Intracellular extracts were obtained either by suspending washed cell pellets in 10 ml of water and heating them for 15 min at 100°C , or by the perchloric acid procedure (Inbar and Lapidot, 1988a). The supernatant was separated by centrifugation at 15,000 x g for 15 min and concentrated under reduced pressure to 1 ml.

(c) Separation of THP(A) and THP(B) from *S. parvulus* cell extracts

Cell extract samples were mixed with 4 volumes of 1 M acetic acid before separation by Dowex 50 W chromatography. The column was washed with deionized water (five times the column volume) to remove carbohydrates and polyols. Amino acids, THP(A) and THP(B) were eluted with 3 M NH_4OH . Ammonia was evaporated to dryness and the residue was brought to pH 5 and separated by Dowex 1 anion exchange (acetate-form) chromatography. Acidic amino acids such as glutamic acid remained attached to the anion exchange column. THP(A) and THP(B) were eluted with water.

(d) Separation of THP(A) and THP(B) from peptides and proteins

The mixture of THP(A) and THP(B) obtained in step (c), was added to a 40 ml column of Sephadex G-25. The column was washed with water. The first fractions consisted of peptides and low molecular weight proteins (yellow in color), and THP(A) and THP(B) were next eluted.

(e) Separation of THP(A) from THP(B).

The mixture of THP(A) + THP(B) obtained in step (d) was mixed with 4 volumes of 1 M acetic acid before adding to a 70 ml column of Dowex 50 W x 8 (NH_4^+ form). THP(A) together with a small amount of THP(B) were eluted with water. THP(B) together with a small amount of THP(A) were displaced with 3 M ammonium hydroxide.

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By repeating these procedures once more, complete separation of THP(A) from THP(B) was obtained. The fraction of THP(B) contained also a small amount of alanine. The latter fraction was brought to pH 2 (with HCl) and separated on a 70 ml column of Dowex 1 anion exchange (OH⁻ form) chromatography. The column was washed with water and THP(B) was displaced with 150 ml of 1% formic acid (alanine was eluted with 600 ml of 1% formic acid). The ¹H NMR spectra of purified THP(A) and THP(B) are shown in Fig. 1 (b) and (a), respectively. The molecular structures are shown in Fig. 2.

Example 2: Crystallization of THP(A) and THP(B).2H₂O

(a) Pure THP(A) obtained according to Example 1 was dissolved in hot methanol and the solution was left in a desiccator under benzene vapor for crystallization. Crystals appeared after 7 days. The transparent, prismatic crystals having the dimensions 0.2x0.1x0.05 mm were collected for X-ray crystal structure analysis. Crystal structure of (C₆H₁₀N₂O₃), trigonal with space group P3₁ was found. The bond lengths and bond angles of the crystals are presented in Tables 1 and 2, respectively. The stereochemical configuration of THP(A) was found to be the S form.

(b) Pure THP(B) obtained according to Example 1 was dissolved in ethanol and a few drops of methylene chloride were added to the solution. The solution was left to evaporate slowly. After a few days, crystals appeared in the solution. The transparent prismatic crystals having the dimensions 0.4x0.3x0.2 mm were collected for X-ray crystal structure analysis. Crystal structure of C₆H₁₀N₂O₃.2[H₂O] is tetragonal with space group P4₃. The bond lengths and bond angles of the crystals are presented in Tables 1 and 2, respectively. The stereochemical configuration of THP(B) was found to be the S form.

Table 1

Bond lengths (Å°)			
THP(B)		THP(A)	
C(2)-N(1)	1.303 (4)	C(2)-N(1)	1.311 (10)
C(2')-C(2)	1.487 (4)	N(3)-C(2)	1.310 (8)
C(4)-N(3)	1.459 (4)	C(4)-N(3)	1.458 (9)
C(5)-C(4)	1.522 (4)	C(4')-C(4)	1.545 (11)
O(8)-C(4')	1.260 (3)	O(9)-C(5)	1.405 (10)
C(6)-N(1)	1.456 (4)	O(8)-C(4')	1.217 (10)
N(3)-C(2)	1.312 (4)	C(6)-N(1)	1.462 (9)
C(4')-C(4)	1.529 (5)	C(2')-C(2)	1.499 (10)
O(7)-C(4')	1.247 (4)	C(5)-C(4)	1.527 (12)
C(6)-C(5)	1.508 (5)	C(6)-C(5)	1.519 (10)
		O(7)-C(4')	1.272 (9)

Table 2

Bond angles (degree)			
THP(B)		THP(A)	
C(6)-N(1)-C(2)	123.7 (3)	C(6)-N(1)-C(2)	121.5 (6)
N(3)-C(2)-N(1)	121.5 (3)	C(2')-C(2)-N(1)	118.1 (5)
C(4)-N(3)-C(2)	122.4 (3)	C(4)-N(3)-C(2)	124.2 (6)
C(5)-C(4)-N(3)	108.9 (3)	C(4')-C(4)-N(3)	113.5 (7)
O(7)-C(4')-C(4)	116.5 (3)	C(6)-C(5)-C(4)	109.4 (7)
O(8)-C(4')-O(7)	126.0 (3)	O(9)-C(5)-C(6)	108.8 (6)
C(5)-C(6)-N(1)	109.1 (3)	O(7)-C(4')-C(4)	111.9 (7)
C(2')-C(2)-C(2')	118.8 (3)	O(8)-C(4')-O(7)	127.8 (7)
N(3)-C(2)-C(2')	119.7 (3)	N(3)-C(2)-N(1)	121.7 (7)
C(4')-C(4)-N(3)	112.9 (3)	C(2')-C(2)-N(3)	120.2 (7)
C(5)-C(4)-C(4')	110.4 (3)	C(5)-C(4)-N(3)	109.5 (6)

Table 2 (continued)

Bond angles (degree)			
THP(B)		THP(A)	
O(8)-C(4')-C(4)	117.5 (3)	O(9)-C(5)-C(4)	111.3 (6)
C(5)-C(5)-C(4)	109.6 (2)	C(5)-C(5)-N(1)	108.6 (6)
		O(8)-C(4')-C(4)	120.2 (6)

Example 3: Binding of Act D to oligonucleotide in the presence of THP(A) and THP(B) - NMR studies

Characterization of the binding of Act D with DNA has been the subject of considerable interest since this is the presumed basis of its antitumor activity. It seems clear that the peptide chains pendant on the phenoxazone ring are largely responsible for the preference of the binding of Act D at GpC sites of DNA. Detailed solution structure modeling investigations have been carried out on the 1:1 complex of Act D and d(ATGCAT) (Lybrand et al., 1986).

The mode of binding of THPs to DNA in the presence and absence of Act D was investigated by ¹H NMR spectroscopy. The oligonucleotide d(ATGCAT) was selected for this study for several reasons: it contains a unique strong binding site for Act D, it is long enough to interact with the pentapeptide lactones of Act D, and the kinetics of Act D-d(ATGCAT) association are slow and mimic the kinetics of Act D-DNA association (Brown et al., 1984; Zhou et al., 1989). ¹H NMR studies conducted on DNA-THP (A) and (B) complexes indicate that THP(A) and THP(B) bind the oligonucleotide and largely prevent its complex formation with Act D.

(a) Binding of THPs to d(ATGCAT). A comparison of the chemical shifts of the non-exchangeable protons in the free d(ATGCAT) and in complexes of d(ATGCAT) with THP(A) and THP(B) are shown in Table 3. On addition of THP(A) and THP(B) to the hexamer, the guanosine H8 (GH8) signal at 7.94ppm exhibited the major downfield chemical shift (20Hz), the adenosine (terminal) H8 protons moved downfield by 10Hz, while all the other base aromatic protons exhibited only minor changes.

(b) Binding of THPs to the Act D-d(ATGCAT) complex. On addition of THP(B) to the complex of Act D-d(ATGCAT) (at a molar ratio of 0.64 Act D per hexamer), the chemical shift of GH8 protons moved downfield by 0.018ppm, while the chemical shift of the adenosine (internal) H2 proton of the Act D-hexamer complex at 7.99ppm remained unchanged (Fig. 3b). When THP(A) was added, an additional downfield shift (0.22ppm) of GH8 protons was noted (Fig. 3c). These results indicate that THPs bind the free oligonucleotide even in the presence of Act D.

(c) Binding of Act D to d(ATGCAT). The binding of Act D to free oligonucleotide and to the oligonucleotide-THPs complex have been investigated by the ¹H NMR titration technique. On addition of 0.16 molar equiv. of Act D to the hexamer, new proton peaks of the complex appeared (Fig. 4b) and their intensities increased up to 1:1 Act D to d(ATGCAT) (Fig. 4a, b, c, d, e). The signal ratios of free hexamer and hexamer bound to Act D are shown in Table 4.

(d) Binding of Act D to the d(ATGCAT)-THPs complex. When 0.16 molar equiv. of Act D was added to the hexamer-THPs complex, no new proton peaks of the Act D-hexamer complex appeared (Fig. 4b'); on addition of 0.32 molar equiv. of Act D, only one proton peak of the Act D-hexamer complex was detectable at 8.03ppm (Fig. 4c'). Only when 0.48 molar equivalent of Act D was added to the hexamer-THPs, could most of the new proton peaks of the Act D-hexamer be distinguished, as shown in Fig. 4d', e'. Two resonances of the Act D-hexamer are missing, the signal of the adenosine (internal) H2 of the Act D-hexamer complex at 7.99ppm could not be detected due to the intense resonance of GH8 of the THPs-hexamer complex at 7.98ppm, while the resonance of adenosine (terminal) H2 at 7.87ppm of the Act D-hexamer complex was obscured by the coincident resonance of the NH proton of THP(B) at 7.88ppm. The equilibrium binding properties of Act D-d(ATGCAT) in the presence and absence of the THPs (Table 4) were derived from the proton peak area ratios of the free hexamer to the bound Act D-hexamer. Due to difficulties in obtaining precise binding data at low values of Act D bound to the hexamer, binding constants could not be determined. However, equilibrium measurements (Fig. 4 and Table 4) demonstrate the potential activity of THPs in protecting DNA from Act D intercalation by some kind of binding to DNA, probably by a mechanism competitive with the antibiotic.

Table 3

Base proton chemical shift assignments (ppm) of nonexchangeable protons of d(ATGCAT) and d(ATGCAT) - THP(A) + THP(B)			
Assign ¹	d(ATGCAT) δ 1	THP(A)+THP(B)-d(ATGCAT) δ 2	Difference δ 1 - δ 2
A ⁸	8.330	8.330	0.000
A ⁶	8.186	8.203	+0.017
G ⁵	7.944	7.984	+0.040
THP(A)		7.913	0.000
A ²	7.908	7.908	0.000
THP(B)		7.881	
A ²	7.844	7.849	+0.005
C ⁶	7.368	7.376	+0.008
T ⁶	7.356	7.356	0.000
T ⁶	7.251	7.256	+0.005
Adenosine (internal) H8 (A ⁸); adenosine (terminal) H8 (A ⁸); guanosine H8 (G ⁸); adenosine (internal) H2 (A ²); adenosine (terminal) H2 (A ²) cytosine H6 (C ⁶); thymine (internal) H6 (T ⁶) and thymine (terminal) H6 (T ⁶).			

Table 4

Equilibrium binding of Act D to d(ATGCAT) in the presence and absence of THPs		
Molar ratio of Act D/d(ATGCAT)	d(ATGCAT) (free/bound)*	d(ATGCAT)-THPs (free/bound)*
0.16	4.9	n.d.
0.32	1.7	3.5
0.48	0.8	2.7
0.64	0.4	1.3

(*) Proton peak area ratios of free hexamer A₁⁸ (two protons/2); bound Act D-hexamer A₁⁸ (one proton). n.d. - non-determined.

Example 4: in vitro transcription studies in the presence of Act D; the protection effect of THP(A) + THP(B)

(a) Transcription inhibition by Act D

The inhibition of RNA polymerase transcription caused by Act D binding to DNA was studied using the system described in Kadesch and Chamberlin, 1982, in which purified RNA polymerases from mammalian cells, such as RNA polymerases I, II and III from HeLa cells, are able to transcribe defined dCMP-tailed DNA templates efficiently and specifically. The dCMP-tailed DNA system allows quantitative studies of transcription reactions by the polymerase protein itself in the absence of other protein factors, and is thus an appropriate system to analyze the direct effect of DNA-binding drugs on the transcription process, and the protective effect of THP(A) and THP(B) on transcription in the presence of Act D.

DNA-dependent RNA polymerases I, II and III from HeLa cells (pol I, pol II and pol III, respectively) were purified by a method which is a modification of the procedure described by Hodo and Blatt, 1977.

The templates DNA were linearized by restriction enzyme Pst I generating a 3' protruding end, and tailed at both ends with dCTP by using terminal transferase (Boehringer Mannheim). Following ethanol precipitation, the dCMP-tailed DNAs were digested with a second restriction enzyme, and the selected tailed fragment was purified from agarose gel as described by Kadesch and Chamberlin, 1982.

Transcription reaction mixtures were incubated in 15 μ l of transcription buffer containing 70 mM Tris-HCl (pH 7.9), 0.1 M NH₄Cl, 6 mM MgCl₂, 5 mM spermidine, 10% glycerol, 0.15 mM dithiothreitol, 0.6 μ g of dCMP-tailed template, and 0.5 μ l of HeLa RNA polymerase (3u) at 30°C for 10 min. Pulse buffer (3.5 μ l) containing 4.3 mM of ATP, GTP and CTP, and 170 μ M UTP, 20 μ Ci of [α -³²P]UTP was added to the transcription reaction mixture for 20 seconds incubation. Transcription elongation then took place by adding 1.5 μ l of chase buffer containing 7 mM UTP in the presence or absence of 0.06 μ g Act D. Transcription elongation was carried out for 10 min and was then stopped by addition of proteinase K buffer (0.6% SDS; 25 mM Tris pH 7.9; 25 mM EDTA final concentration) 200 μ g/ml proteinase K, and 30

µg of tRNA, and incubated for 25 min at 65°C. The sample was ethanol precipitated, pellet was resuspended in loading buffer (90% formamide; 0.5 x Tris borate buffer) and subjected to 6% polyacrylamide - urea gel electrophoresis. The autoradiogram of the gel was analyzed by molecular dynamics computing densitometer to determine the relative intensities of the bands. (Nucleoside triphosphates and tRNA were purchased from Sigma, proteinase K from Boehringer Mannheim, and [α - 32 P]CTP (400 Ci/mmol) from Amersham International, United Kingdom).

The sensitivity of the three RNA polymerases from HeLa cells to various concentrations of Act D was determined, using the purified polymerases and a dCMP-tailed template of 225 bp for pol I and of 420 bp for pol II and pol III. These templates are expected to give runoff transcripts of 225 and 420 nucleotides, respectively, since RNA polymerase initiates transcription at discrete sites at the single-stranded tail double stranded duplex junction. In all experiments a pulse-chase protocol was used.

The transcription inhibition patterns due to Act D of the three polymerases (pol I, II and III, respectively) are summarized in Table 5 and indicate that the enzymes are similarly sensitive to Act D. When 1 µg/ml Act D is added with the chase, only 10-20% residual transcription occurs, yet at a concentration of 3-5 µg/ml only 0.1-3% residual transcription is obtained.

These findings are in good agreement with the notion that Act D inhibition is dependent on the DNA concentration in the reaction mixture.

Table 5

Percentage of residual transcription in the presence of Act D					
RNA Polymerase	Actinomycin-D µg/ml				
	0	1	3	4	5
I	100	16			3
II	100	22		3	3
III	100	13	3		0.1

(b) Resumption of Act D inhibited transcription by THP(A) and THP(B)

The assay was performed in a concentration of Act D causing over 97% inhibition of transcription, that was taken as 3 µg/ml. It was figured that the concentration of the THP(A) + THP(B) required to prevent binding of Act D to DNA would be higher than that of Act D, since they are expected to protect the DNA in general and not necessarily at d (GpC) base pair (bp), whereas the concentration of Act D is related to the d(GpC) content of the DNA. As can be seen in lanes 2, 6, 10 of Fig. 5 and in Table 6, in these experiments THP(A) and THP(B) by themselves did not inhibit the transcription reactions. In the presence of either THP(A) alone or THP(B) alone, the inhibition of transcription caused by Act D was not affected (as shown in Fig. 5, lanes 3-5 and 7-9, and in Table 6). Yet when THP(A) and THP(B) were added together in a ratio of 1:1, their effect was dramatic. At a concentration of 200 µM there was a recovery of 75% of transcription in the presence of 2.5 µM Act D, which by itself would have inhibited 97% of the reaction. These results indicate that the combination of THP(A) and THP(B) have a synergistic effect on protection of DNA from Act D binding to the DNA. The conditions described above indicate that inhibition of transcription (97%) is obtained by one molecule of Act D per 30 bp, whereas saturation of DNA-Act D complex formation would have required a ratio of 1:10. In order to recover 75% transcription, 80 molecules of THP(A) + THP(B) per each molecule of Act D are required, or a ratio of one pair of THP(A) + THP(B) per bp. These results suggest the role of THP(A) + THP(B) in protection of DNA against Act D binding, which enables transcription to be resumed in the presence of Act D.

Table 6

Residual transcription in the presence of Act D and THP(A) and THP(B)					
Lane No.		THP		Act D [3µg/ml] [2.5 µM]	% transcription
		µg/ml	µM		
1	control	0	0	-	100
2	THP(A)	60	400	-	100
3	THP(A)	10	66	+	0.2
4	THP(A)	30	200	+	0.5
5	THP(A)	60	400	+	1.5
6	THP(B)	60	400	-	100

Table 6 (continued)

Residual transcription in the presence of Act D and THP(A) and THP(B)					
Lane No.		THP		Act D [3µg/ml] [2.5 µM]	% transcription
		µg/ml	µM		
7	THP(B)	10	60	+	1.0
8	THP(B)	30	200	+	2.7
9	THP(B)	60	400	+	5.1
10	THP(A+B)(1:1)	60	400	-	100
11	THP(A+B)(1:1)	10	66	+	4.6
12	THP(A+B)(1:1)	30	200	+	75.0
13	THP(A+B)(1:1)	0	0	+	0.2

Example 5. Cell growth and drug treatment : Reversal of adriamycin inhibition of DNA synthesis due to THPs.

HL60 cells were grown in RPMI medium (Gibco) containing 10% fetal calf serum (Biological Laboratories - Bot Haemek) in microwell plates (Nunc). Cells were suspended in medium at a concentration of 2×10^5 cells per ml and dispersed into the wells of the plates in 100 µl aliquots. Following 20 hrs of incubation at 37°C, the cells were treated with THPs (100 µg/ml) and adriamycin at increasing concentrations as indicated in Fig. 6. After 24 hrs [3 H] thymidine was added (1 µCi/well, 120 µl) for a duration of 3 hrs. Thymidine incorporation was stopped by addition of 25 µl trichloroacetic acid (TCA) at 20%, and the plates were placed at 4°C overnight. TCA precipitates were washed with 10% TCA (100 µl), solubilized in 100 µl 1N NaOH and transferred to vials for counting in the presence of 3 ml scintillation solution containing toluene:Lumax (Lumac, The Netherlands) (3:2) mix. Assays were performed in triplicates.

As can be seen in Figure 6, when HL60 cells are treated with adriamycin at concentrations of 0.01-0.02 µg/ml [3 H] thymidine incorporation is inhibited by 30-40%, respectively. Addition of THPs (A+B) (at a ratio of 1:1) at final concentration of 100 µg/ml reverses the inhibitory effect of adriamycin so that in spite of the presence of adriamycin (0.01-0.02 µg/ml) [3 H] thymidine incorporation is very efficient, 107%-93%, respectively. This result gives further indication that the THPs may protect DNA from damaging drugs.

Example 6. In vitro transcription using HeLa whole cell extract (WCE) : Reversal of actinomycin D inhibition of transcription due to the presence of THPs.

WCE was prepared according to Manley, 1984. A 20-µl reaction contained 10 µl WCE, 1 µg DNA, 4mM creatine phosphate, 500 µM ATP, GTP, UTP, 50 µM CTP, 20 µM Cl of [α - 32 P]CTP, and transcription was performed at 30°C. All reactions were preincubated 20 min before the addition of the nucleotide mixture; 3 min after the addition of radioactive nucleotides, (pulse), chase mix containing 10 mM unlabelled CTP was added and transcription proceeded for additional 20 min. Reactions were stopped and RNA was analyzed as in Example 4.

In a preliminary experiment, the degree of transcription inhibition by actinomycin D in the HeLa-WCE system was checked. A concentration of 0.4 µg/ml was used, which gave about 80% inhibition. In the experiment presented in Figure 7, it is clear that Act D alone at 0.4 µg/ml inhibits the transcription reaction by 80% (compare lane 0 to lane C). Addition of low concentrations of THPs do not affect the reaction up to 20 µg/ml, whereas at concentrations of 100-200 µg/ml there is resumption of transcription up to 40-65% of the control. This experiment indicates that THPs (A+B) may reverse the Act D inhibition of transcription resuming transcription by about 25%-45% above the residual 20% of transcription caused by actinomycin D alone.

These results further indicate that THPs may protect the DNA from drugs that bind to it. Thus THPs cause reversal of the inhibitory effects of DNA binding drugs and enable efficient nucleic acid synthesis by polymerases in spite of the presence of the drugs.

Example 7. DNA protection by THPs from digestion by restriction enzymes.

Further evidence of the functional interaction of THPs and DNA stems from our finding that a mixture of THP(A) and THP(B), and THP(A) alone, are able to protect a plasmid DNA (pGEM10, Promega) from digestion by restriction endonucleases (EcoRI, Aval and DraI). The THPs interact with DNA and effectively (at 10^{-4} M) protect the cleavage sites recognized by the restriction endonucleases. The results are summarized in Table 7.

THPs were added to the digestion mixture to a final concentration as indicated in Table 7. One µg of pGEM10 was subjected to digestion with 0.2-1 µl of enzyme in a final volume of 20 µl buffer as recommended by the supplier (New

England Biolabs) for 3hrs. At the end of the incubation time, 5 μ l of stop buffer containing 75% glycerol, 3% bromophenol blue and 10 mM EDTA were added and 12 μ l of the reaction mix were loaded onto 1% agarose gel. The gel was stained with ethidium bromide (0.5 μ g/ml), visualized by UV and photographed. The relative intensities of the bands in the photographs were analyzed by molecular dynamics computing densitometry.

The minimal amount of THP(A) + THP(B) needed to protect DNA from digestion by restriction endonucleases is in the range of 25 to 100 μ g/ml, whereas 150 to 250 μ g/ml was needed for complete inhibition. From the comparison of Aval and DraI in the presence of both THP(A) and (B), it seems that Aval activity is 4 times more sensitive to the inhibitory effect of the THPs. This finding is probably the result of high affinity of THPs to GC residues of Aval sites in comparison to AT residues of DraI sites (see Table 7). The range of THP(A) + (B) concentration needed to protect DNA from endonuclease digestion is comparable to the amounts of actinomycin D and distamycin A that bind to DNA, as described before (Nosikov et al., 1976 and 1977).

It is noteworthy that while DNA is protected by THPs from digestion by restriction endonucleases, the activity of RNA polymerase is not affected either by THP(A) or THP(B), or both, as shown by the inventors of the present invention. This difference may arise from the mode of binding of the two enzymes. RNA polymerase binds to duplex DNA and searches for a promoter site by forming transient hydrogen bonds with exposed hydrogen donor and acceptor groups of base pairs, whereas EcoRI endonuclease recognizes its palindrome target site without breaking base pairs but through electrostatic attraction to the phosphate backbone of the DNA.

Table 7 Protection of the DNA by THIP's from restriction enzymes' attack

Restriction Endonuclease	Restriction site	No. of sites	THIP(A)		THIP(A)+(B)		Relation of $\frac{\text{THIP(A)}}{\text{THIP(A)+(B)}}$
			partial $\mu\text{g/ml}$	complete mM	partial $\mu\text{g/ml}$	complete mM	
EcoRI	5'-GAATTC	1	100	0.66	100	0.66	4
	3'-CTTAAG					250	
AvaI	5'-CCCGGG	1	25	0.166	300	1.98	2
	3'-GGGCCC				25	0.166	
DraI	5'-TTTAAA	3	100	0.66	300	1.98	2
	3'-AAATTT				100	0.66	

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Claims

- 25 1. A pharmaceutical composition comprising as active ingredient at least one tetrahydropyrimidine derivative selected from 2-methyl-4-carboxy-5-hydroxy-3,4,5,6-tetrahydropyrimidine [THP(A)], a salt, a 5-ether or a 5-ester thereof, and 2-methyl-4-carboxy-3,4,5,6-tetrahydropyrimidine [THP(B)] or a salt thereof, and a pharmaceutically acceptable carrier.
- 30 2. A pharmaceutical composition according to claim 1 comprising 2-methyl-4-carboxy-5-hydroxy-3,4,5,6-tetrahydropyrimidine [THP(A)].
- 35 3. A pharmaceutical composition according to claim 1 comprising 2-methyl-4-carboxy-3,4,5,6-tetrahydropyrimidine [THP(B)].
4. A pharmaceutical composition according to any of claims 1 to 3 comprising THP(A) and THP(B).
- 40 5. A pharmaceutical composition according to claim 4 wherein THP(A) and THP(B) are present in equimolar amounts.
6. A pharmaceutical composition according to any of claims 1 to 5 for protection of DNA in non-tumor tissues from damage by DNA-binding drugs.
7. A pharmaceutical composition according to any of claims 1 to 5 for decreasing the toxic effects of an antitumor antibiotic in cancer chemotherapy.
- 45 8. A pharmaceutical composition according to claim 7 wherein the antitumor antibiotic is actinomycin D.

Patentansprüche

- 50 1. Arzneimittel, umfassend als Wirkstoff mindestens ein Tetrahydropyrimidin-Derivat, ausgewählt aus 2-Methyl-4-carboxy-5-hydroxy-3,4,5,6-tetrahydro-pyrimidin[THP(A)], einem Salz, einem Ether in 5-Stellung oder einem Ester in 5-Stellung davon und 2-Methyl-4-Carboxy-3,4,5,6-tetrahydropyrimidin[THP(B)] oder einem Salz davon, und einen pharmazeutisch verträglichen Träger.
- 55 2. Arzneimittel nach Anspruch 1, umfassend 2-Methyl-4-carboxy-5-hydroxy-3,4,5,6-tetrahydropyrimidin[THP(A)].

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3. Arzneimittel nach Anspruch 1, umfassend 2-Methyl-4-carboxy-3,4,5,6-tetrahydropyrimidin[THP(B)].
4. Arzneimittel nach einem der Ansprüche 1 bis 3, umfassend THP(A) und THP(B).
5. Arzneimittel nach Anspruch 4, worin THP(A) und THP(B) in equimolaren Mengen enthalten sind.
6. Arzneimittel nach einem der Ansprüche 1 bis 5 zum Schutz der DNA in Nicht-Tumorgeweiben vor Schädigung durch DNA-bindende Arzneistoffe.
7. Arzneimittel nach einem der Ansprüche 1 bis 5 zur Verringerung der toxischen Wirkung eines Anti-Tumor-Antibiotikums in der Krebs-Chemotherapie.
8. Arzneimittel nach Anspruch 7, worin das Anti-Tumor-Antibiotikum Actinomycin D ist.

Revendications

1. Composition pharmaceutique comprenant comme ingrédient actif au moins un dérivé de tétrahydropyrimidine choisi parmi la 2-méthyl-4-carboxy-5-hydroxy-3,4,5,6-tétrahydropyrimidine [THP(A)], un de ses sels, un éther en 5 ou un ester en 5, et la 2-méthyl-4-carboxy-3,4,5,6-tétrahydropyrimidine [(THP(B))] ou un de ses sels, et un support pharmaceutiquement acceptable.
2. Composition pharmaceutique selon la revendication 1 comprenant la 2-méthyl-4-carboxy-5-hydroxy-3,4,5,6-tétrahydropyrimidine [(THP(A))].
3. Composition pharmaceutique selon la revendication 1 comprenant la 2-méthyl-4-carboxy-3,4,5,6-tétrahydropyrimidine [THP(B)].
4. Composition pharmaceutique selon l'une quelconque des revendications 1 à 3 comprenant la THP(A) et la THP(B).
5. Composition pharmaceutique selon la revendication 4 dans laquelle la THP(A) et la THP(B) sont présentes en quantités équimolaires.
6. Composition pharmaceutique selon l'une quelconque des revendications 1 à 5 pour la protection de l'ADN dans des tissus non tumoraux contre les dommages causés par les médicaments qui se lient à l'ADN.
7. Composition pharmaceutique selon l'une quelconque des revendications 1 à 5 pour diminuer les effets toxiques d'un antibiotique antitumoral dans la chimiothérapie du cancer.
8. Composition pharmaceutique selon la revendication 7 dans laquelle l'antibiotique antitumoral est l'actinomycine D.

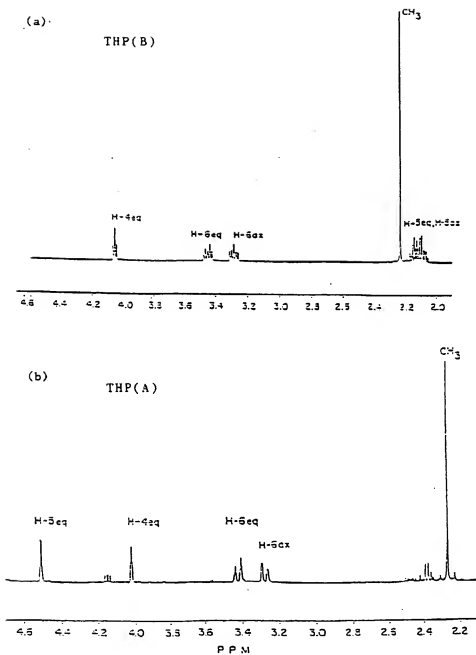


Figure 1

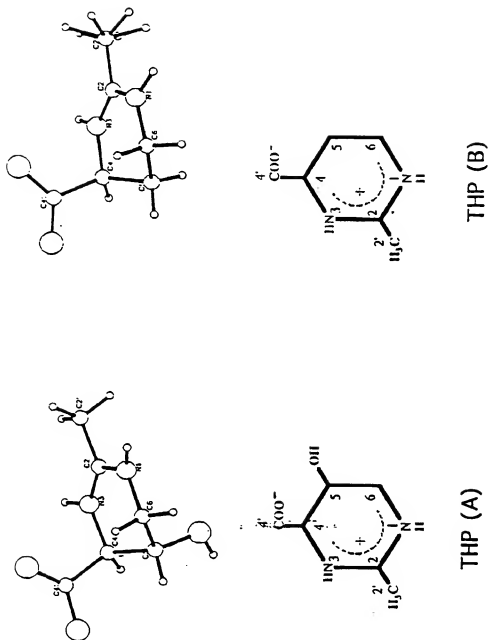


Figure 2

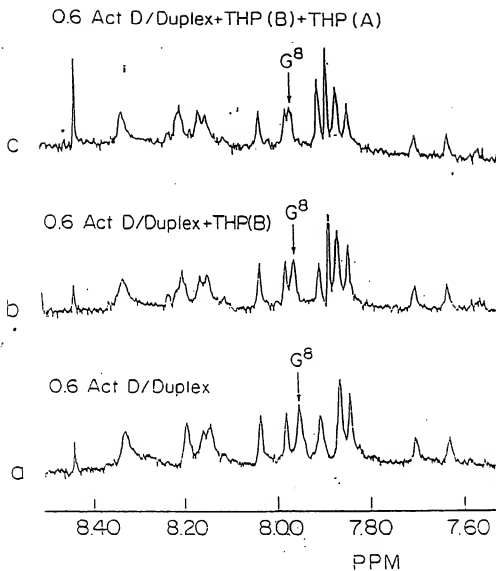


Figure 3

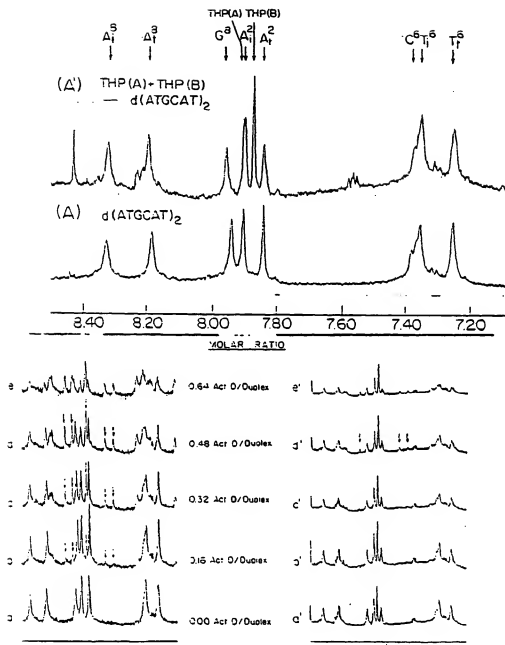


Figure 4

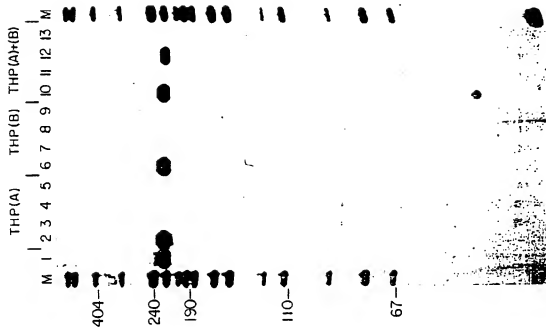
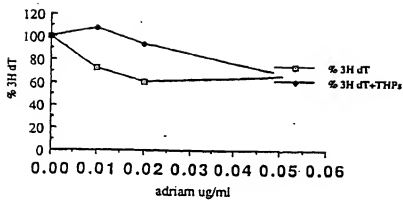


Figure 5



	adriam ug/ml	% 3H dT	% 3H dT+THPs
1	0.000	100.000	100.000
2	0.010	72.000	107.000
3	0.020	60.000	93.000
4	0.050	65.000	88.000

Figure 6

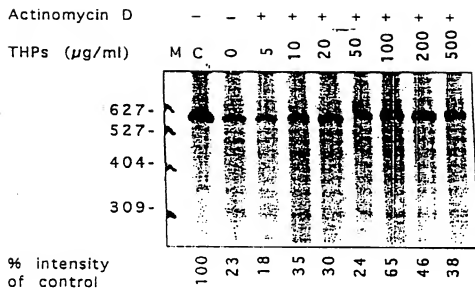


Figure 7

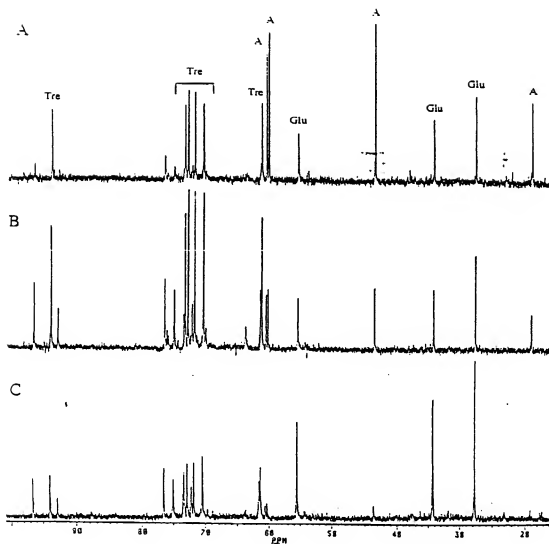


Figure 8

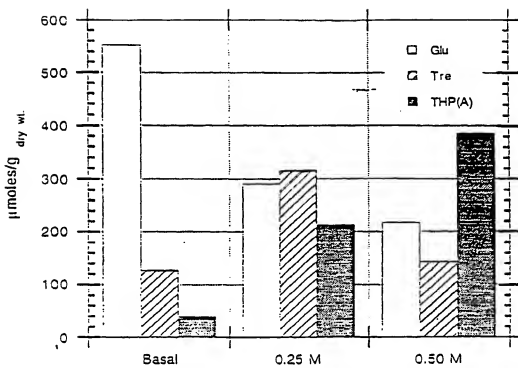


Figure 9

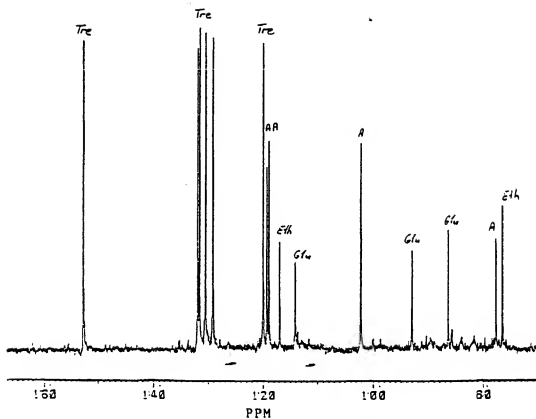


Figure 10.

Figure 11

